REMARKS

Rejections under 35 U.S.C.§112, first paragraph

The Examiner maintains the rejection of claims 1, 3 and 4 under 35 U.S.C. §112, first paragraph with the assertion that the specification does not provide support for a genus encompassing kits comprising either three nucleic acids or three cofactors as the first through third affinity reagents. Applicant traverses this rejection and withdrawal thereof is respectfully requested.

Originally filed claim 3 recites, "wherein the affinity reagents are lectins, receptors, single chain antibodies, cofactors and nucleic acids." Thus, original claim 3 provides support for a genus encompassing kits comprising three nucleic acids or three cofactors. The specification has been amended on page 3 to provide the corresponding description in the specification. The amendment to the specification in no way adds new matter because the amended material is the same as original claim 3.

In addition, "a specification is directed to those skilled in the art and need not teach or point out in detail that which is well-known in the art." In re Nelson, 280 F.2d 172, 126 USPQ 242 (Ct. Cust. & Pat. App. 1960). Specific examples of proteins, which have three binding sites would be readily recognized by those skilled in the art and need not be listed by the Applicants; for example all

antibodies typically have at least three binding sites with the variable regions and the Fc portion, IgM has at least 10 binding sites, and capsid proteins on viral surfaces have many binding sites. Withdrawal of the rejection is respectfully requested.

Rejections under 35 U.S.C. §112, second paragraph

The Examiner maintains the rejection of claims 1, 3 and 4 under 35 U.S.C.§112, second paragraph as being indefinite. More specifically, the Examiner asserts that it is not clear what three nucleic acids or cofactors might bind to the same protein at the same time. As noted above, specific examples of proteins, which have three binding sites would be readily recognized by those skilled in the art. In addition, the purpose of the requirements of 35 U.S.C. §112, second paragraph "is to ensure that the scope of the claims is clear so the public is informed of the boundaries of what constitutes infringement of the patent." M.P.E.P. §2173. The present claims clearly defines the boundaries of the invention as requiring three affinity reagents each of which binds to a different site on a protein macromolecule. One skilled in the art would readily be able to determine whether a particular test kit falls within the scope of the claims and would infringe the patent by the simple test of whether there are three different reagents present which bind to

different sites on the macromolecule being assayed. As such, the requirements for 35 U.S.C. §112, second paragraph, are fully met.

Rejections under 35 U.S.C.§102(b)

The Examiner maintains the rejection of claims 1, 3, 4 and 5 under 35 U.S.C. §102(b) as being anticipated by Landegren et al. (U.S. Pat. No. 4,988,617). Applicant traverses this rejection and withdrawal thereof is respectfully requested.

In the Office Action of January 16, 2002, the Examiner asserts that the claims encompass affinity reagents that bind to a macromolecule, which is a nucleic acid, and that recitation that the macromolecule is "a protein" does not define the affinity reagents. Applicant believes that the Examiner has misinterpreted the claims with this position because the claims recite that the reagents have affinity for a macromolecule and further require that the macromolecule is a protein; thus the reagents must have affinity for a protein. However, claim 1 has been amended as follows to make the binding relationship more clear between the affinity reagents and macromolecule more clear.

> *1. A test kit comprising*
> *a) a first immobilized reagent having* ***specific*** *affinity to a* ***protein*** *macromolecule, and*
> *b) a second and a third affinity reagent specific for different determinants of said* ***protein*** *macromolecule, and modified with conjugatable oligonucleotides which conjugate through*

i) hybridization of an oligonucleotide complementary to the conjugatable oligonucleotides;
ii) hybridization of the conjugatable oligonucleotides to each other; or
iii) ligation of the oligonucleotides,
wherein a signal is generated by nucleic acid amplification only when said second and third affinity reagents are closely bound on said ***protein*** *macromolecule.*

The Examiner further asserts that Applicant's argument that the reagents of Landegren et al. are not composed of separate parts are insufficient. The Examiner interprets the nucleic acids of Landegren et al. having "two parts." The Examiner's position appears to be that the end of the nucleic acid of Landegren et al. that binds the macromolecule is a first part and the end of the nucleic acid that is conjugatable is the second part even though Landegren et al. never refer to the process as being made from two parts. As discussed above, the claims have been amended to more clearly define the invention as requiring affinity reagents that are specific for protein macromolecules. As such, the affinity reagents of Landegren et al., which specifically bind other oligonucleotides, do not fall within the present claims.

Thus, the present invention is not anticipated by Landegren and withdrawal of the rejection is, therefore, respectfully requested.

Rejections under 35 U.S.C.§103

The Examiner further maintains the rejection of claims 6 and 8-10 as being obvious over Cantor et al. (U.S. Pat. No. 5,635,602) combined with Suzuki et al. and of claims 1-6 and 8-10 as being obvious over Cantor et al. combined with de la Monte et al. (U.S. Pat. No. 5,830,670). Applicant traverses these rejections and withdrawal thereof is respectfully requested.

The Examiner asserts that Applicant's arguments that with Cantor et al. the second and third reagents are added with the oligonucleotides being "pre-conjugated" are insufficient because the claims do not recite that the second and third reagents are added separately. Applicant believes that the Examiner may have somewhat misunderstood the arguments in this regard of September 21, 2001. It appears that the Examiner interpreted the discussion in the September 21, 2001 response that the first and second reagents are separate, as meaning that the first and second reagents are added in separate steps, not that they are added as separated reagents, as was intended.

As discussed in the September 21, 2001 response, Cantor et al. discloses an assay where two reagents with affinity for a target protein have already been connected via double stranded DNA sequences when they are added to a sample. With the present invention, the second and third affinity reagents each are connected to a single DNA

strand and are added as separate reagents to the immobilized target molecule. With the assay of the present invention, the conjugation of the second and third affinity reagents after binding indicates the juxtaposition of the two target determinants.

As a result of the second and third affinity reagents being completely dissociated upon addition, with the invention, there is almost no background signal because most non-specific binding events will not result in the generation of a signal. At the same time, specific signal can be amplified. These advantages are not possible with the assay of Cantor et al. because with Cantor et al. once one of the affinity probes becomes immobilized there is an increased risk that the connected probe will also become immobilized even if there is no correct target determinant, increasing the background signal. In addition, with Cantor et al. before the oligonucleotides can be amplified, all the connected proteins must first be separated using restriction digestion of the oligonucleotide duplex. Any non-specifically bound complex that is not cleaved will serve as a template for amplification further creating non-specific background signal. The problems associated with Cantor et al. significantly reduce the assay sensitivity and problems associated with cleaving the connecting olignonucleotide duplex are discussed in column 13, line 51 of Cantor et al.

Claim 6 has been amended as indicated below to further clarify the distinction from Cantor et al. that leads to the improvements associated with the present invention.

> *6. An immunoassay for detection of a specific antigen, comprising:*
> *a) contacting a sample suspected of containing said specific antigen with a first antibody linked to a solid support, said first antibody being specific for a first epitope on the antigen;*
> *b) washing off excess sample;*
> *c) incubating with a solution of a second and a third antibody specific for a second and a third epitope of said antigen, and modified with conjugatable oligonucleotides, wherein* ***said second and third antibodies are separate from each other in an unbound state and*** *said oligonucleotides conjugate to each other when said second and third antibody are both bound to said antigen through...*

The Examiner further asserts that the method of Cantor et al. will result in reduced non-specific binding. As discussed above, by adding the second and third affinity reagents as separate, dissociated reagents, the present invention can reduce non-specific binding. The reduction in non-specific binding associated with the present invention is not possible advantages with the assay of Cantor et al. because with Cantor et al. affinity reagents are joined together prior to addition to the assay.

In the response of September 21, 2001 Applicant relied on a manuscript prepared by Fredriksson et al. as evidence of the unexpected results associated with the present invention. The manuscript has

since published in Nature and a copy of the corresponding published article is attached hereto. The data of Figure 4 of the manuscript corresponds to Figure 6 of the manuscript. The Examiner asserts that the data of Figure 4/Figure 6 is not probative because the Figure was not in the originally filed specification. However, under M.P.E.P. §706.02(g) publications may be relied on as evidence of unexpected results. The Examiner also raises the issue that the data of Figure 4 was obtained using specific reagents and that the claims are not limited to these reagents. However the unexpected improved properties associated with the present invention result from the use of three affinity reagents that all bind to different sites, wherein two of the affinity reagents have been modified with conjugatable oligonucleotides that will only conjugate to one another after the affinity reagents bind to the target protein. The unexpected properties demonstrated in Figure 6 of the Fredricksson et al. publication, would be expected by one skilled in the art to be observed with any combination of affinity reagents used provided that the affinity reagents bind to three independent sites on the target protein. As such, the data of Figure 6 is fully commensurate with the scope of the invention and demonstrates the unexpected properties associated with the claimed invention.

Finally, the Examiner asserts that Suzuki et al. suggests modifying immunoPCR assays with an immobilized antibody to improve results and that de la Monte et al. teach the use of an immobilized antibody and immunoPCR in disease diagnosis. As discussed above, the improved sensitivity and reduced back ground associated with the present invention results from the use of three independent affinity reagents that bind to separate sites on the target protein, wherein two of the affinity reagents have been modified with conjugatable oligonucleotides that will only conjugate to one another after the affinity reagents bind to the target protein. None of the references teach the premise for the invention regarding the conjugation of the nucleic acids on the second and third affinity reagents after binding. As such, the invention is neither disclosed nor suggested by the references and cannot be achieved if the references are combined in their teachings. Withdrawal of the rejections is therefore respectfully requested.

As the above-presented amendments and remarks in no way add new matter or raise new issues for consideration and further address and overcome the rejections of the Examiner, reconsideration and allowance of the claims are respectfully requested. Should the Examiner have any questions regarding the present application, she is requested to

contact MaryAnne Armstrong, PhD (Reg. No. 40,069) in the Washington DC area, at (703) 205-8000.

Pursuant to 37 C.F.R. §§ 1.17 and 1.136(a), Applicant(s) respectfully petition(s) for a one (1) month extension of time for filing a reply in connection with the present application, and the required fee of $55.00 is attached hereto.

If necessary, the Commissioner is hereby authorized in this, concurrent, and future replies, to charge payment or credit any overpayment to Deposit Account No. 02-2448 for any additional fees required under 37 C.F.R. §§1.16 or 1.17; particularly, extension of time fees.

Respectfully submitted,

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Attachments

MARKED-UP VERSION SHOWING CHANGES

IN THE CLAIMS

Claims 1 and 6 have been amended as follows:

1. (Five times amended) A test kit comprising

a) a first immobilized reagent having **specific** affinity to a ~~specific~~ **protein** macromolecule, and

b) a second and a third affinity reagent specific for different determinants of said **protein** macromolecule, and modified with conjugatable oligonucleotides which conjugate through

i) hybridization of an oligonucleotide complementary to the conjugatable oligonucleotides;

ii) hybridization of the conjugatable oligonucleotides to each other; or

iii) ligation of the oligonucleotides,

wherein a signal is generated by nucleic acid amplification only when said second and third affinity reagents are closely bound on said **protein** macromolecule~~; wherein said macromolecule is a protein~~.

6. (Four times amended) An immunoassay for detection of a specific antigen, comprising:

a) contacting a sample suspected of containing said specific antigen with a first antibody linked to a solid support, said first antibody being specific for a first epitope on the antigen;

b) washing off excess sample;

c) incubating with a solution of a second and a third antibody specific for a second and a third epitope of said antigen, and modified with conjugatable oligonucleotides, wherein **said second and third antibodies are separate from each other in an unbound state and** said oligonucleotides conjugate to each other when said second and third antibody are both bound to said antigen through

i) hybridization of an oligonucleotide complementary to the conjugatable oligonucleotides;

ii) hybridization of the conjugatable oligonucleotides to each other; or

iii) ligation of the oligonucleotides;

d) washing off excess solution;

e) amplifying said conjugated oligonucleotides; and

f) detecting the amplified products.

Protein detection using proximity-dependent DNA ligation assays

Simon Fredriksson[1], Mats Gullberg[1], Jonas Jarvius[1], Charlotta Olsson[1], Kristian Pietras[2], Sigrún Margrét Gústafsdóttir[1], Arne Östman[2], and Ulf Landegren[1]*

The advent of *in vitro* DNA amplification has enabled rapid acquisition of genomic information. We present here an analogous technique for protein detection, in which the coordinated and proximal binding of a target protein by two DNA aptamers promotes ligation of oligonucleotides linked to each aptamer affinity probe. The ligation of two such proximity probes gives rise to an amplifiable DNA sequence that reflects the identity and amount of the target protein. This proximity ligation assay detects zeptomole (40×10^{-21} mol) amounts of the cytokine platelet-derived growth factor (PDGF) without washes or separations, and the mechanism can be generalized to other forms of protein analysis.

Sensitive and specific methods are required to study concentration, location and colocalization, and secondary modification of the large sets of proteins that are predicted from genome sequence data—stringent demands that are difficult to meet with current techniques. By contrast, methods in use for detection of specific nucleic acid sequences, such as PCR, offer excellent sensitivity and specificity[1]. This is only partially a consequence of the strong and predictable affinity between complementary nucleic acid strands. Of greater importance is the requirement that pairs of probes recognize a given target sequence, and that only this dual interaction can initiate an amplified detection signal. Such a requirement for dual and proximate recognition is also a frequent element of cellular recognition reactions in signal transduction and gene regulation[2].

We have developed an analogous method for *in vitro* analysis of proteins and other macromolecules, termed proximity ligation. The method depends on the simultaneous and proximate recognition of target molecules by pairs of affinity probes, giving rise to an amplifiable detection signal. The molecule we targeted was the homodimer of the platelet-derived growth factor B-chain (PDGF-BB), a cytokine with growth- and differentiation-promoting effects[3]. As affinity probes we used so-called DNA aptamers, a class of oligonucleotide reagents obtained through a process of *in vitro* selection for affinity to a target molecule[4,5], and shown to be suitable for analytical applications[6,7]. A DNA aptamer with affinity for PDGF-BB (ref. 8) was extended by additional sequence elements at either the 5′ or the 3′ end, forming a proximity probe pair. When pairs of probes bind PDGF-BB, the free ends of their sequence extensions are brought sufficiently close to hybridize together to a subsequently added connector oligonucleotide, allowing the ends to be joined by enzymatic DNA ligation. The detected protein molecules thus promote the ligation reaction by bringing together the free ends of the proximity probe pairs. The ligation products can then be replicated by nucleic acid amplification through PCR, while unreacted probes remain silent (Fig. 1).

Results

Detection of PDGF-BB by proximity ligation. On the basis of crude molecular modeling, we selected DNA extensions of the affinity probes of around 40 nucleotides each. However, we found that the length of the extensions could be varied over a considerable range with negligible effects on ligation efficiency and nonspecific signal (data not shown). The method thus also has the potential to allow analysis of large proteins.

The connector oligonucleotide was designed to base-pair to ten nucleotides at the free end of each member of the proximity probe pair. A short hybridizing length and 5′ and 3′ end sequences that do not base-pair to the proximity probes were selected to prevent the connector oligonucleotide from giving rise to ligation-independent amplification products by acting as a primer and/or template for amplification. Longer connector oligonucleotides of 14 + 14 or 16 + 16 hybridizing nucleotides yielded false PCR products (data not shown). The connector oligonucleotide was added in large molar excess over proximity probes to ensure efficient formation of ligation substrates.

We carried out homogeneous assays for PDGF-BB, requiring no washes or separations, by first preincubating proximity probes with the samples at room temperature for 1 h or at 37°C for 15 min. We then added a mixture that included all reagents required for both probe ligation and quantitative PCR with real-time detection[9], thereby also diluting the reaction. Finally, we transferred the reactions to a fluorometric PCR instrument for detection of ligated molecules.

To optimize assay parameters and investigate reaction mechanisms, we preincubated a proximity probe pair with a fixed amount of PDGF-BB or with no PDGF-BB. Then we added variable amounts of connector oligonucleotide and allowed it to act as template for the ligation of proximity probe pairs for 5, 25, or 125 min before PCR with real-time detection (Fig. 2A). With addition of high concentrations of connector oligonucleotide, colocalized ends of proximity probes were joined rapidly and extended incubation resulted in little increase in either signal or background. This lack of further ligation at high connector concentrations renders the assay quite robust and

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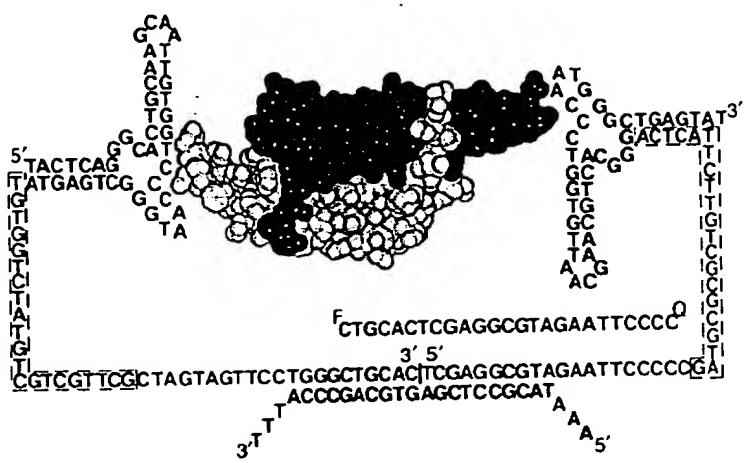

Figure 1. Schematic view of the homodimeric PDGF-BB (ref. 23) bound by two aptamer-based proximity probes, A1 and A2, for detection by proximity ligation. The sequence of the aptamer 41t specific for the PDGF B-chain[8] is shown in black, sequence extensions to be joined by ligation upon hybridization to a common connector oligonucleotide are shown in blue (A1) and red (A2), and primer sites for PCR are boxed. A probe for real-time detection of PCR products via the 5'-nuclease assay is shown in green with a 5' FAM fluorophore and a 3' TAMRA quencher.

probably reflects the hybridization of nearly all free proximity probes to one connector oligonucleotide each, inhibiting the formation of new ligation substrates. A connector oligonucleotide concentration of 400 nM was selected for subsequent experiments, and ligation was allowed to proceed for 5 min before amplification.

Next, we varied preincubation volumes and concentrations of proximity probes. All samples were then brought to a final volume of 50 µl by adding the ligation and amplification mix (Fig. 2B). Increased detection sensitivity resulted if PDGF-BB-independent ligation events were limited by using a minimal amount of probes, and by preincubating the proximity probes and sample in the smallest possible volume to promote binding. In further experiments we used 20 pM proximity probes in a preincubation volume of 5 µl. The very low probe concentration during the ligation phase of the assay keeps target-independent ligation events to a minimum, and requires a mere 100 attomoles of proximity probes per assay.

By assuming that the ends of oligonucleotide pairs that have bound a protein molecule are constrained within a sphere of a radius defined by the size of the protein (~60 Å) and taking into account the maximal length of the nucleotide extensions (5 Å per nucleotide), we estimate that under these experimental conditions local concentration of ligatable ends of the proximity probes increases by a factor of at least 10^6 upon target binding.

As few as 24,000 PDGF-BB molecules were reproducibly detected over background plus two standard deviations by proximity ligation, ~1,000-fold fewer than were detected by a sandwich enzyme-linked immunosorbent assay (ELISA) for the same protein (Fig. 3). The linear range for quantitation extended over a >1,000-fold concentration range, with sufficient precision to distinguish 2-fold dilutions of the protein. The error associated with the analysis is of the same order as that of quantitative PCR with real-time detection alone, calculated for the number of amplicons generated: 35% coefficient of variation (CV) for proximity ligation (averaged in Fig. 3) and 32% CV for 88 replicates of the diluted amplicon analyzed by real-time PCR.

Under the reported conditions, approximately 1 PDGF molecule in 25 had bound 2 different proximity probes that were subsequently joined by ligation. The reason that not all protein molecules trigger a ligation reaction is probably a consequence of several factors: most importantly, at 20 pM concentrations of proximity probe, well below the reported K_d for the aptamers (~100 pM), only a minority of proteins are expected to have bound one proximity probe, and even fewer will have bound two. Moreover, only one in two such complexes of target protein with two proximity probes involve probe pairs with one 5' end and one 3' end available for ligation. Finally, the ligation efficiency of juxtaposed proximity probe ends could be <100% as a result of imperfections of oligonucleotide synthesis.

The aptamers have a reported 500-fold lower affinity for the PDGF A-chain compared with the B-chain, and accordingly the PDGF-AA homodimer was not detected in the assay and neither were the PDGF-BB homologs PDGF-CC (ref. 10) and PDGF-DD (refs 11,12) (Fig. 3). Furthermore, the PDGF-AB heterodimer was not detected, demonstrating that the assay is truly proximity dependent. The detection reaction could be specifically inhibited through the addition of a fragment of the PDGF β-receptor[13], known to compete with the aptamer for binding to PDGF-BB (ref. 8). PDGF-BB was also analyzed in the presence of fetal calf serum (FCS), cerebrospinal fluid (CSF), and Eagle's minimal essential medium (EMEM) to demonstrate the applicability of the assay in realistic biological samples and cell culture medium (Fig. 4). Closely similar results were obtained in biological samples and in defined media (compare Figs 3 and 4).

The concentration of PDGF-BB in a human serum sample was demonstrated by homogeneous proximity ligation to be 0.2 nM (6 ng/ml), compared with a value of 0.13 nM as determined by ELISA. By contrast, the PDGF-BB content in the corresponding plasma sample was <1 pM as determined by ELISA and <0.2 pM using proximity ligation. The proximity ligation assay further revealed a concentration of 1 pM PDGF-BB in conditioned medium from a 36 h culture of the human anaplastic thyroid carcinoma cell line SW-1736 (ref. 14), shown by northern blot analysis to

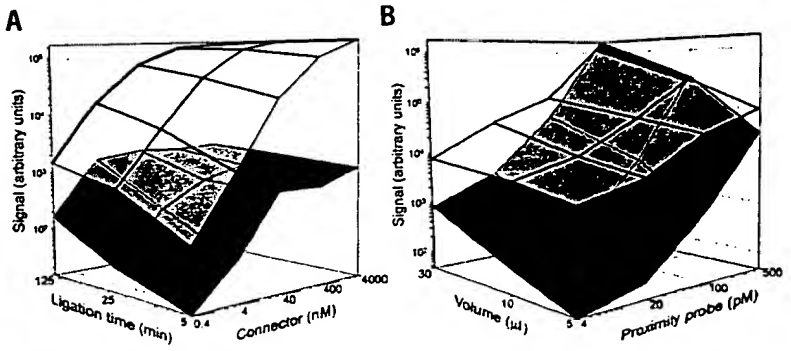

Figure 2. Optimization of reaction conditions for the proximity ligation assay. (A) Effect of connector oligonucleotide concentration and time of ligation on signal and background. (B) Influence of preincubation volume and probe concentration. The upper, yellow layers connect the signals recorded in reactions with 5.6 attomoles of PDGF-BB, while the lower, red layers represent the target-independent background, expressed in arbitrary units. Averages of duplicate measurements are shown.

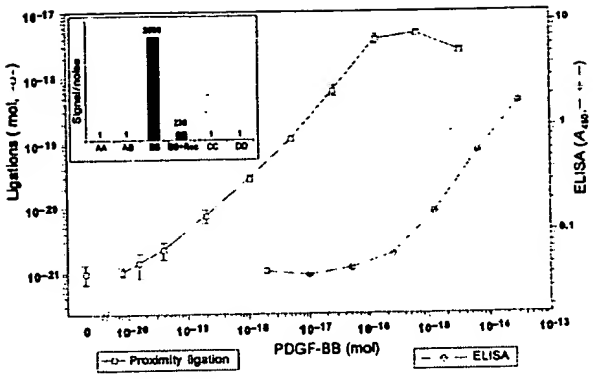

Figure 3. Detection of a dilution series of PDGF-BB by proximity ligation (solid line) and by sandwich ELISA (dashed line). Average values of triplicate and duplicate determinations, respectively, are shown with s.e.m. In the inset panel, results are shown of analyses of 3 fmol PDGF-AA, -AB, -BB, -BB with 1.5 pmol of the PDGF β-receptor, PDGF-CC, and -DD.

transcribe the PDGF-B gene (N. Heldin, personal communication). This corresponds to an estimated production of 1,500 cytokine molecules per cell per day.

Proximity ligation detection of human α-thrombin. We also designed proximity probes for detection of human α-thrombin. A pair of DNA aptamers binding two distinct sites[15,16] were extended with sequences for ligation and amplification to form a proximity probe pair (Fig. 5). The sensitivity of detection illustrates that the proximity ligation strategy is also effective when using affinity probes for two distinct determinants on one protein, and that the procedure should also be useful for detecting interactions between different protein molecules.

Solid-phase proximity ligation. The homogeneous proximity ligation assay is simple to standardize and to carry out for large sets of samples. It eliminates the need for extensive, carefully controlled washes as required in solid-phase assays. However, for samples containing potential inhibitors of ligation or amplification, or in which the concentration of analyte is exceedingly low, solid-support immobilization of the protein can serve both to purify and to concentrate the samples. It also permits removal of unbound probes through washes before the ligation step, further reducing target-independent probe ligation. By immobilizing PDGF-BB through binding to antibody-coated reaction wells, we

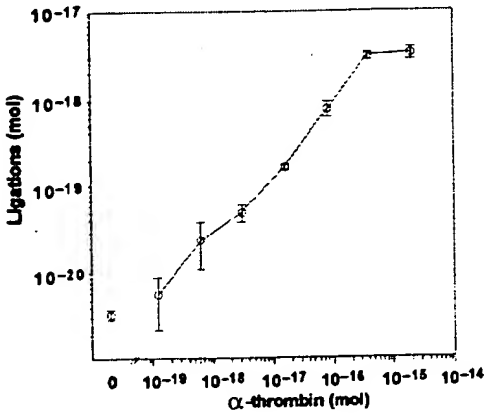

Figure 5. Detection of human α-thrombin by homogeneous proximity ligation. The *x*-axis indicates molar amounts of protein. Averages of duplicate measurements are shown with s.e.m.

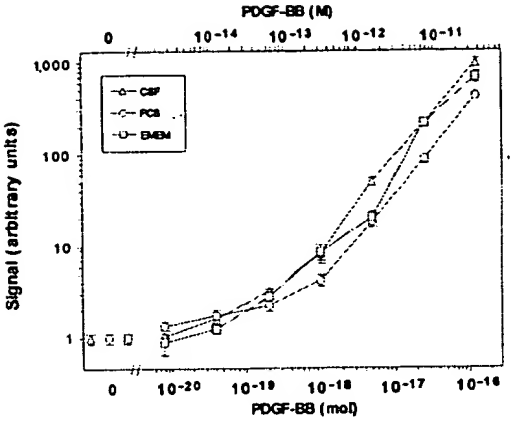

Figure 4. Homogeneous detection of PDGF-BB in the presence of complex biological fluids and cell culture media: FCS, EMEM, and human CSF. The top *x*-axis indicates concentration of PDGF-BB, and the corresponding molar amounts are shown at the bottom *x*-axis. Signals are normalized to a scale of arbitrary units of duplicate measurements with indicated s.e.m.

detected low-femtomolar concentrations of the protein using the same pair of proximity probes as was used in the homogeneous assay (Fig. 6). This compares with a low-picomolar detection threshold in the ELISA (Fig. 3).

PCR has previously been used in solid-phase protein assays for sensitive detection of single affinity probes labeled with DNA strands (immuno-PCR; ref. 17). To compare this strategy with proximity ligation, variable amounts of PDGF-BB were bound to antibodies immobilized in microtiter wells. This permitted us to compare detection of binding by a single aptamer having an oligonucleotide extension amplifiable by PCR, with the use of a proximity probe pair that could be amplified only upon ligation (Fig. 6). Across a range of probe concentrations and washing conditions, immuno-PCR resulted in a considerably higher background, and thereby a reduced sensitivity compared with proximity ligation. In the immuno-PCR approach, any nonspecifically bound probes can contribute to background signals, whereas proximity ligation requires proximity of pairs of probes to yield a signal.

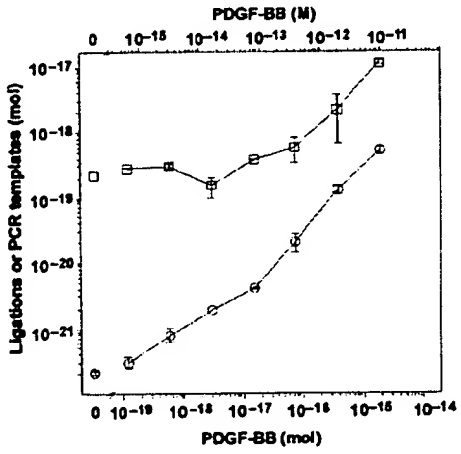

Figure 6. Dilution series of PDGF-BB analyzed in a solid-phase assay, either by proximity ligation (circles) or by aptamer-based immuno-PCR (squares). The top *x*-axis indicates concentration of PDGF-BB analyzed with the corresponding molar amounts at the bottom *x*-axis. Results are represented as averages of duplicate measurements with s.e.m.

Discussion

There are advantages to using aptamers in proximity ligation in that the attachment of DNA extensions is trivial, isolation of aptamers can be automated and scaled up[18], and high-affinity reagents are regularly obtained, as demonstrated in a recent study of 100 protein-specific aptamers[19]. Moreover, this class of affinity probes can be shared in the research community by publishing the nucleotide sequence of appropriate reagents, permitting standardization of protein assays between labs. Nonetheless, most currently available reagents for protein binding are themselves proteins. Preliminary results demonstrate that monoclonal antibodies are suitable as proximity probes after coupling of oligonucleotides (M.G., unpublished results), and that heteroantisera, affinity probes prepared by phage/ribosome display techniques[20], or other types of affinity reagents should also be useful.

The proximity-dependent ligation assay converts the detection of any macromolecule for which suitable pairs of affinity probes exist into a more convenient reaction for detecting nucleic acids. The use of a DNA reporter for the detection reactions permits simultaneous analysis of large sets of proteins by encoding tag sequences in the amplified segment for separate detection, for example by hybridization to a standard oligonucleotide array. Proximity ligation should also enhance the sensitivity and specificity of localized *in situ* detection of proteins, and it could provide a valuable means to investigate secondary modifications of specific proteins, as well as the interaction of pairs of biomolecules in macromolecular complexes.

Experimental protocol

Detection of PDGF-BB by proximity ligation. Unless otherwise indicated, 1 µl aliquots, containing 5.6 attomoles PDGF-BB diluted in 137 mM NaCl, 10.1 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4, 2.7 mM KCl, 1 mM $MgCl_2$, and 1% BSA, were added to optical tubes (Applied Biosystems (ABI), Foster City, CA) containing 20 pM of the proximity probes A1 and A2 in a total volume of 5 µl (50 mM KCl, 10 mM Tris-HCl, pH 8.3, 3.3 mM $MgCl_2$, 0.1% BSA). Upon addition of the combined mix for ligation and amplification, the samples contained 50 mM KCl, 10 mM Tris-HCl, pH 8.3, 1.5 mM $MgCl_2$, 0.4 units T4 DNA ligase (Amersham Biosciences, Uppsala, Sweden), 400 nM connector oligonucleotide, 80 µM ATP, carboxy-x-rhodamine (ROX) internal fluorescence standard, 0.2 mM dNTPs, 0.5 µM primers (forward: 5′-ATGTGGTCTATGTCGTCGTTCG-3′; reverse: 5′-TGAGTAAGAACAGCGCGCAT-3′), 50 nM probe for the 5′ nuclease assay, and 1.5 units AmpliTaq Gold polymerase (ABI) in 50 µl. After a 5 min ligation reaction at room temperature, the reactions were transferred to the real-time PCR instrument for temperature cycling: 10 min at 95°C, and then 15 s at 95°C and 60 s at 60°C, repeated 45 times (ABI PRISM 7700). Signal-to-noise values represent the number of detected ligation products divided by the number of ligation products arising in a sample without PDGF-BB. The number of ligation products was calculated from a standard curve of diluted amplicons.

In experiments for which the concentration of connector oligonucleotide was varied, we took precautions to avoid an influence by the complementarity between the 5′ nuclease probe and the connector oligonucleotide. After a preincubation with proximity probes in a 5 µl volume, a 35 µl ligation mix was added and the ligation reaction was terminated by heating at 95°C for 10 min, followed by the addition of reagents for PCR to a final volume of 50 µl. Furthermore, separate standard curves of diluted amplicons were prepared for each connector concentration.

PDGF-BB detection in biological samples. Before use, FCS was heated to 65°C for 20 min to inactivate the α2-macroglobulin that binds PDGF-BB, blocking the binding of proximity probes[21]. Reactions (5 µl, with 3.4 µl sample and 20 pM proximity probes) were incubated at 37°C for 20 min with 16 µg/ml poly(A) oligonucleotide. In the PCR, $MgCl_2$ (3.5 mM) was used.

PDGF-BB quantitation in biological samples. PDGF-BB was quantified in serum and plasma by adding known amounts of PDGF-BB to a diluted sample and calculating the endogenous concentration from the increase in signal. The specificity of the assay was confirmed by blocking probe binding with an excess of soluble PDGF β-receptor, bringing the signal to background levels. The ELISA was done according to the supplier's instructions (R&D Systems, Minneapolis, MN). The incubation mix also contained 16 µg/ml poly(A) oligonucleotide and PCR was done with 3.5 mM $MgCl_2$.

PDGF-BB expression by the SW-1736 cell line, grown in 2 ml EMEM supplemented with 1% FCS, was assayed by homogeneous proximity ligation of 1 µl samples, diluted 5-fold in PBS with 0.1% BSA, and quantified by reference to analyses of known concentrations of the protein diluted in growth medium. A total of 300,000 cells were plated and after 36 h, 514,000 cells were counted. Specificity of protein detection was confirmed by blocking the signal with an excess of PDGF β-receptor.

Detection of thrombin by proximity ligation. Experimental procedures were identical to those for analysis of PDGF-BB except as indicated below. The proximity probes were Thr1 (5′-CAGTCCGTGGTAGGGCAGGTTGGGGTGACTTCGTCGGAACTATCTAGCGGTGTACGTGAGTGGGCATGTAGCAAGAGG-3′) and Thr2 (5′-P-GTCATCATTCGAATCGTACTGCAATCGGGTATTAGGCTAGTGACTACTGGTTGGTGAGGTTGGGTAGTCACAAA-3′), aptamer sequences underlined. The connector oligonucleotide was 5′-AAGAATGATGACCCTCTTGCTAAAA-3′, and the 5′ nuclease probe was 5′-TET TGTACGTGAGTGGGCATGTAGCAAGAGG-3′-TAMRA (TET, tetrachlorofluorescein; TAMRA, carboxytetramethylrhodamine). Samples were diluted in 100 mM 2-(*N*-morpholino)-ethanesulfonic acid (MES) buffer, pH 6.5, and 1% BSA, and preincubated with proximity probes Thr1 (15 pM) and Thr2 (20 pM) in 100 mM NaCl, 50 mM Tris-HCl, pH 7.5, 1 mM $MgCl_2$. The PCR buffer contained 1.9 mM $MgCl_2$. Primers for PCR were 5′-GTGACTTCGTGGAACTATCTAGCG-3′ and 5′-AATACCCGATTGCAGTACGATTC-3′.

Detection of PDGF-BB by solid-phase proximity ligation and immuno-PCR. An anti-PDGF antiserum immunoglobulin fraction[22] (500 ng) in a 20 µl volume was added to optical tubes for real-time PCR, followed by blocking with 1% BSA. Up to 200 µl of sample was then incubated together with 5 nM of proximity probes or with 0.1 nM of immuno-PCR reagent. Unbound probes were removed by washes (200 µl) using a multichannel pipette (proximity ligation 8 times with 3 flushes per wash, immuno-PCR 8 times with 4 flushes per wash (washes 1 and 2, PBS with 0.02% SDS; washes 3–7, PBS; wash 8, H_2O)). A ligation mix (50 mM KCl, 10 mM Tris-HCl, pH 8.3, 1.5 mM $MgCl_2$, 0.15 mM ATP, 50 nM connector oligonucleotide, 2 U T4 DNA ligase in 20 µl) was added and the reactions were kept at 30°C for 5 min and then at 80°C for 20 min. Next, a PCR mix was added to a final volume of 50 µl, containing ROX internal fluorescence standard, 0.1 mM dNTPs, 0.2 µM primers, 50 nM probe for the 5′ nuclease assay, and 1 unit AmpliTaq Gold polymerase.

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1. Saiki, R.K. *et al.* Enzymatic amplification of β-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. *Science* **230**, 1350–1354 (1985).
2. Hartwell, L.H., Hopfield, J.J., Leibler S. & Murray A.W. From molecular to modular cell biology. *Nature* **402**, c47–52 (1999).
3. Heldin, C.H. & Westermark, B. Mechanism of action and *in vivo* role of platelet-derived growth factor. *Physiol. Rev.* **79**,1283–1316 (1999).
4. Tuerk, C. & Gold, L. Systematic evolution of ligands by exponential enrichment: RNA ligands to bacteriophage T4 DNA polymerase. *Science* **249**, 505–510 (1990).
5. Ellington, A.D. & Szostak, J.W. *In vitro* selection of RNA molecules that bind specific ligands. *Nature* **346**, 818–822 (1990).
6. Jayasena, S.D. Aptamers: an emerging class of molecules that rival antibodies in diagnostics. *Clin. Chem.* **45**, 1628–1650 (1999).
7. Ellington, A.D. & Robertsson, M.P. *In vitro* selection of an allosteric ribozyme that transduces analytes to amplicons. *Nat. Biotechnol.* **17**, 62–66 (1999).
8. Green, L.S. *et al.* Inhibitory DNA ligands to platelet-derived growth factor B-chain. *Biochemistry* **45**, 14413–14424 (1996).
9. Held, C.A., Stevens, J., Livak, K.J. & Williams, P.M. Real time quantitative PCR. *Genome Res.* **6**, 986–994 (1996).
10. Li, X. *et al.* PDGF-C is a new protease-activated ligand for the PDGF α-receptor. *Nat. Cell Biol.* **2**, 302–309 (2000).
11. Bergsten, E. *et al.* PDGF-D is a specific, protease-activated ligand for the PDGF β-receptor. *Nat. Cell Biol.* **5**, 512–516 (2001).
12. LaRochelle, W.J. *et al.* PDGF-D, a new protease-activated growth factor. *Nat. Cell Biol.* **5**, 517–521 (2001).
13. Leppänen, O. *et al.* Predimerization of recombinant platelet-derived growth factor receptor extracellular domains increases antagonistic potency. *Biochemistry* **39**, 2370–2375 (2000).
14. Dahlman, T. *et al.* Fibrosis in undifferentiated (anaplastic) thyroid carcinomas: evidence for a dual action of tumor cells in collagen type I synthesis. *J. Pathol.* **191**, 376–386 (2000).
15. Macaya, R.F. *et al.* Structural and functional characterization of potent antithrombotic oligonucleotides possessing both quadruplex and duplex motifs. *Biochemistry* **34**, 4478–4492 (1995).
16. Tasset, D.M., Kubik, M.F. & Steiner, W. Oligonucleotide inhibitors of human thrombin that bind distinct epitopes. *J. Mol. Biol.* **272**, 688–698 (1997).
17. Sano, T., Smith, C.L. & Cantor, C.R. Immuno-PCR: very sensitive antigen detection by means of specific antibody-DNA conjugates. *Science* **258**,120–122 (1992).
18. Drolet, D.W., Jenison, R.D., Smith, D.E., Pratt, D. & Hicke, B.J. A high throughput platform for systematic evolution of ligands by exponential enrichment (SELEX). *Comb. Chem. High Throughput Screen.* **2**, 271–278 (1999).
19. Brody, E.N. *et al.* The use of aptamers in large arrays for molecular diagnostics. *Mol. Diagn.* **4**, 381–388 (1999).
20. Li, M. Applications of display technology in protein analysis. *Nat. Biotechnol.* **18**, 1251–1256 (2000).
21. Bonner, J.C. & Osornio-Vargas, A.R. Differential binding and regulation of platelet-derived growth factor A and B chain isoforms by α2-macroglobulin. *J. Biol. Chem.* **27**, 16236–16242 (1995).
22. Thyberg, J., Ostman, A., Backstrom, G., Westermark, B. & Heldin, C.H. Localization of platelet-derived growth factor (PDGF) in CHO cells transfected with PDGF A- or B-chain cDNA: retention of PDGF-BB in the endoplasmic reticulum and Golgi complex. *J. Cell Sci.* **97**, 219–229 (1990).
23. Oefner, C., D'Arcy, A., Winkler, F.K., Eggimann, B. & Hosang, M. Crystal structure of human platelet-derived growth factor BB. *EMBO J.* **11**, 3921–3926 (1992).